RYEGRASS POLLEN SPECIFIC PROMOTERS AND EXPRESSION CONTRUCTS

The present invention relates generally to an isolated nucleic acid molecule capable of modifying tissue-specific expression, preferably of a second nucleic acid molecule operably linked thereto. More particularly, the present invention is directed to an isolated nucleic acid molecule, capable of modifying pollen-specific expression, preferably of an operably linked second nucleic acid molecule. The present invention further contemplates constructs including the molecule and methods of using the molecule, including for example, the modification of gene expression in pollen, such as *via* down- or up-regulation, and the introduction into pollen of desired phenotypes.

Forage grasses are the backbone of sustainable agriculture and contribute extensively to the world economy. Two related genera, *Festuca* (fescues) and *Lolium* (ryegrasses) are of significant value in temperate grasslands. These genera contain well-adapted, very productive grasses widely distributed in temperate and cool climates in North and South America, Europe, Asia, Australia and New Zealand, where they are used for agricultural and recreational purposes (Jauhar 1993). The commercially most important ryegrasses in cool temperate climates throughout the world are Italian or annual ryegrasses. In New Zealand and Australia, perennial ryegrass is grown on more than 10 million ha providing high quality forage to support over 60 million sheep and cattle (Siegel *et al.* 1985). However, ryegrasses and other forage species are also responsible for a major portion of grass pollen allergies worldwide. Pollen allergy, in particular grass pollen allergy, is a major environmental disease that afflicts about 20% of the population in cool temperate climates.

Accordingly, there is a need for a means for the generation of useful agronomic plants having modified pollen-specific gene expression, for example plants that are *inter alia* male sterile and/or that produce low allergenic pollen.

In one aspect, the present invention provides an isolated nucleic acid molecule including a sequence of nucleotides selected from the group consisting of (a) a nucleotide sequence set forth in SEQ ID NO:2 or 3; (b) a sequence which

hybridises to SEQ ID NO:2 or 3 under moderately stringent or high stringency conditions; (c) a complement of (a) or (b); and (d) a fragment or variant of (a), (b) or (c);

wherein said molecule is capable of modifying pollen-specific expression, preferably of an operably-linked second nucleic acid molecule.

The nucleic acid molecule may be obtained from ryegrass (*Lolium*) or fescue (*Festuca*) species. These species may be of any suitable type, including Italian or annual ryegrass, perennial ryegrass, hybrid ryegrass, tall fescue, meadow fescue and red fescue. Preferably the species is a ryegrass, more preferably perennial ryegrass (*L. perenne*). The nucleic acid molecule may also be a synthethic molecule.

A pollen-specific promoter has been isolated from *Lolium perenne*, which promoter shows useful properties for targeted pollen-specific expression. Such promoters are particularly useful in the production of low pollen allergen transgenic plants, for transgene containment and/or for the down-regulation of the expression of genes that are involved in pollen development to produce, for example, male sterile or infertile plants.

The modification of pollen-specific gene expression has many uses in plant breeding and development. For example, while the potential of biotechnology in the development of improved plant cultivars is now well recognised, the possibility for transgene escape to wild and non-transformed species raises commercial and ecological concerns. Accordingly, one possible use is in the development of mechanisms whereby transgenic plants are caused to be male infertile, thereby reducing the potential for cross-pollination with other, non-transgenic plants. The regulation of male fertility in plants also has other applications; for example, in the maintenance of uniformity and hybrid vigour of F1 hybrid plants by ensuring that self-pollination is minimised during seed production.

In other instances, it may be desirable to modify pollen-specific gene expression in order to reverse sterility.

In still other cases, it may be desirable to generate hybrids, for example by crossing a plant, which has been caused to be male infertile, with another fertile plant, upon which has been conferred pollen-specific expression of a desired trait.

Another possible use relates to the high pollen allergen production of certain plant species. The ability to modify pollen gene expression would permit manipulation of the production of pollen allergens by plants, thereby facilitating the development of plants causing reduced pollen allergenicity.

As used herein, the term "isolated" means that the material is removed from its original environment (eg. the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid fragment present in a living plant is not isolated, but the same nucleic acid fragment separated from some or all of the coexisting materials in the natural system, is isolated. Such an isolated nucleic acid fragment could be part of a vector and/or such nucleic acid fragments could be part of a composition, and still be isolated in that such a vector or composition is not part of its natural environment.

The term "isolated" also encompasses synthetic molecules, for example of a hybrid or modular promoter.

By "variant" in respect of a nucleotide sequence is meant, for example, an analogue, derivative or mutant, which remains capable of modifying pollen-specific expression, preferably of an operably-linked second nucleic acid molecule.

Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the nucleotides are contemplated so long as the modifications do not result in loss of functional activity of the variant. Preferably the variant has at least approximately 80% identity, such as for example 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88% or 89% identity to the relevant part of the above mentioned sequence, more preferably at least approximately 90%, such as for example 91%, 92%, 93% or 94%, identity, most preferably at least approximately 95% identity, such as for example 96%, 97%, 98%, 99% and 100% identity.

The present invention also extends to variants of the nucleic acid molecule of the present invention, which variants are from a ryegrass (Lolium) or fescue (Festuca) species, and which variants have a corresponding coding region with at least approximately 80% identity, such as for example 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88% or 89% identity, more preferably at least approximately 90% identity, such as for example 91%, 92%, 93% or 94%, identity, most preferably at least approximately 95% identity, such as for example 96%, 97%, 98%, 99% and 100% identity to the coding sequence shown in Fig 1 hereto.

By "fragment" in respect of a nucleotide sequence is meant a part of the nucleic acid molecule, which remains capable of modifying pollen-specific expression, preferably of an operably-linked second nucleic acid molecule. Such fragments may have a size of, for example, at least approximately 15 nucleotides, at least approximately 30 nucleotides, at least approximately 45 nucleotides, at least approximately 100 nucleotides, or at least approximately 200 nucleotides.

In a particularly preferred embodiment the fragment may include one or more sequences selected from the groups consisting of:

AGGTCA (Zm13 Q element; SEQ ID NO:4);

TGTGGTTATATA (LAT52 element; SEQ ID NO: 5); and

GTGA (GTGANTGIO element; SEQ ID NO:6).

By "operably-linked" is meant that the nucleic acid molecule of the present invention is capable of causing expression of a second or further nucleic acid molecule in a plant cell. Usually, the nucleic acid molecule is upstream of the second or further nucleic acid molecule. Where a terminator is operably-linked, the terminator is capable of terminating the expressed transcript of the second or further nucleic acid molecule. Usually, the terminator is downstream of the second or further nucleic acid molecule.

By "expression" is meant that a relevant nucleic acid molecule is transcribed and optionally translated. Thus, the term "expression" can relate both

to the transcription of ribonucleic acid (RNA) from the DNA, as well as the transcription of RNA followed by the translation of that RNA into an amino acid sequence. Modifying expression includes, for example the situation where the nucleic acid molecule of the present invention is used to down-regulate expression of an endogenous gene, for example using anti-sense or sense suppression technology, or interfering RNA (RNAi) or hairpin approaches. Modifying expression also includes use of the nucleic acid molecule of the present invention to express a protein encoded by an existing endogenous gene or to introgress an exogenously-derived sequence and optionally express protein therefrom.

By "pollen-specific" is meant that the expression is substantially confined to the pollen.

"Moderately stringent conditions" or "high stringency conditions" for hybridization may be identified as described by Sambrook *et al*, 1989, the relevant disclosure of which is incorporated herein by reference.

Such conditions are readily determinable by a person skilled in the art, and are generally an empirical calculation based on probe length, salt concentration and washing temperature. For example, the use of a washing solution including approximately 0.7 to approximately 0.2 x SSC (standard sodium citrate), at approximately 50°C to approximately 60°C, would generally be considered moderately stringent conditions. For example, the use of a washing solution including approximately 0.2 to approximately 0.1 x SSC at approximately 60°C to approximately 70°C would generally be considered high stringency conditions.

Reference herein to a "gene", "second nucleic acid molecule" or "further nucleic acid molecule" is to be taken in its broadest context and includes a deoxyribonucleic acid (DNA) sequence which is capable of having its expression modified by the nucleic acid molecule of the present invention. As referred to above, the term "expression" can relate both to the transcription of ribonucleic acid (RNA) from the DNA, as well as the transcription of RNA followed by the translation of that RNA into an amino acid sequence. Accordingly, a gene, second or further nucleic acid molecule includes within its scope both a DNA coding for an

amino-acid encoding RNA (i.e. mRNA) as well as a DNA encoding a RNA that does not code for an amino acid sequence. Such an RNA that does not code for an amino acid sequence may include an antisense RNA. A gene, second or further nucleic acid molecule may be of a wild-type or altered form. In the case of an altered form, the sequence may be modified by alterations to the nucleotide sequence.

In a preferred embodiment of this aspect of the invention, the further nucleic acid molecule is a sequence, for example a gene or fragment thereof, capable of modifying expression of a pollen allergen, preferably capable of causing down-regulation of expression of a pollen allergen. Preferably the pollen allergen is *Lol p* 1 and/or *Lol p* 2.

In a preferred embodiment of this aspect of the invention, the isolated nucleic acid molecule includes the nucleotide sequence shown in SEQ ID NO:2 or 3 or a fragment or variant thereof. In a particularly preferred embodiment, the fragment or variant may include the nucleotide sequence shown in SEQ ID NO:2 or 3, modified so that the final nucleotide in the terminal 3' nucleotide sequence CCAGA is deleted and the penultimate nucleotide in that sequence is modified such that the sequence is CCAC.

In a particularly preferred embodiment the fragment may include one or more sequences selected from the groups consisting of:

AGGTCA (Zm13 Q element; SEQ ID NO:4);

TGTGGTTATATA (LAT52 element; SEQ ID NO: 5); and

GTGA (GTGANTGIO element; SEQ ID NO:6).

In a further aspect of the present invention there is provided a construct including a nucleic acid molecule according to the present invention.

The term "construct" as used herein refers to an artificially assembled or isolated nucleic acid molecule, which includes the nucleic acid molecule of the

present invention. In general a construct may also include further nucleic acid molecule(s) of interest, a marker gene which in some cases may also be the further nucleic acid molecule of interest and other appropriate regulatory sequences. It should be appreciated that the inclusion of these other regulatory sequences in the construct is optional, for example, such sequences may not be required in situations where the regulatory sequences of a host cell are to be used. The term construct includes vectors but should not be seen as being limited thereto. The term construct also includes chimeric genes.

The term "vector" as used herein encompasses both cloning and expression vectors. Vectors are often recombinant molecules containing nucleic acid molecules from several sources.

Accordingly, the construct may be a vector. In a preferred embodiment of this aspect of the invention, the vector may include a further nucleic acid molecule, for example a gene or fragment thereof, a nucleic acid molecule according to the present invention and a terminator; said nucleic acid molecule, further nucleic acid molecule and terminator being operably-linked.

In a preferred embodiment of this aspect of the invention, the further nucleic acid molecule is a sequence, for example a gene or fragment thereof, capable of modifying expression of a pollen allergen, preferably capable of causing down-regulation of expression of a pollen allergen. Preferably the pollen allergen is *Lol p* 1 and/or *Lol p* 2.

In another embodiment, the vector may include more than one further nucleic acid molecule. The further nucleic acid molecules within the same vector may have identical or differing sequences. In a particularly preferred embodiment, each further nucleic acid molecule has one or more upstream nucleic acid molecules according to the present invention and one or more downstream terminators, although expression of more than one further nucleic acid molecule from an upstream nucleic acid molecule or termination of more than one further nucleic acid molecule from a downstream terminator(s) is not precluded.

The vector may be of any suitable type and may be viral or non-viral. The vector may be an expression vector. Such vectors include chromosomal, non-chromosomal and synthetic nucleic acid sequences, eg. derivatives of plant viruses; bacterial plasmids; derivatives of the Ti plasmid from *Agrobacterium tumefaciens*, derivatives of the Ri plasmid from *Agrobacterium rhizogenes*; phage DNA; yeast artificial chromosomes; bacterial artificial chromosomes; binary bacterial artificial chromosomes; vectors derived from combinations of plasmids and phage DNA. However, any other vector may be used as long as it is replicable, or integrative or viable in the plant cell.

The terminator may be of any suitable type and may be endogenous to the target plant cell or may be exogenous, provided that it is functional in the target plant cell. A variety of terminators which may be employed in the vectors of the present invention are well known to those skilled in the art. The terminator may be from the original genomic sequence from which the promoter sequence was isolated or a different genomic sequence. Particularly suitable terminators are polyadenylation signals, such as the CaMV 35S polyA and other terminators from the nopaline synthase (*nos*) and the octopine synthase (*ocs*) genes.

The vector may include further elements necessary for expression of the further nucleic acid molecule, in different combinations, for example vector backbone, origin of replication (ori), multiple cloning sites, spacer sequences, enhancers, introns [such as the maize Ubiquitin Ubi intron), antibiotic resistance genes and other selectable marker genes (such as the neomycin phosphotransferase (*npt2*) gene, the hygromycin phosphotransferase (*hph*) gene, the phosphinothricin acetyltransferase (*bar* or *paf*) gene], and reporter genes [such as beta-glucuronidase (GUS) gene (*gusA*)]. The vector may also contain a ribosome binding site for translation initiation. The vector may also include appropriate sequences for amplifying expression.

As an alternative to use of a selectable marker gene to provide a phenotypic trait for selection of transformed host cells, the presence of the vector in transformed cells may be determined by other techniques well known in the art,

such as PCR (polymerase chain reaction), Southern blot hybridisation analysis, histochemical GUS assays, northern and western blot hybridisation analyses.

Those skilled in the art will appreciate that the various components of the vector are operably-linked, so as to result in expression of said further nucleic acid molecule(s) gene or genes. Techniques for operably linking the components of the vector of the present invention are well known to those skilled in the art. Such techniques include the use of linkers, such as synthetic linkers, for example including one or more restriction enzyme sites.

The construct may be a chimeric gene. Accordingly, in a further aspect of the present invention there is provided a chimeric gene including a nucleic acid molecule of the present invention operably linked to further nucleic acid molecule(s) capable of causing down-regulation of expression of a pollen allergen, for example a gene or genes encoding one or more pollen allergens or a fragment thereof. Preferably, the pollen allergens are the major pollen allergens *Lol p* 1 and/or *Lol p* 2. The sequence of the further nucleic acid molecule may be in either a sense or antisense orientation when operably linked with the nucleic acid molecule of the present invention. In a preferred embodiment, the chimeric gene is included in a vector which may be used to transform a plant cell.

The constructs, vectors and chimeric genes of the present invention may be incorporated into a variety of plants, including: monocotyledons, such as grasses from the genera *Lolium*, *Festuca*, *Paspalum*, *Pennisetum*, *Panicum* and other forage and turfgrasses, corn, rice, sugarcane, oat, wheat and barley; dicotyledons, such as arabidopsis, tobacco, soybean, canola, cotton, potato, chickpea, medics, white clover, red clover, subterranean clover, alfalfa, eucalyptus, poplar, and hybrid aspen; and gymnosperms, such as pine tree. In a preferred embodiment, the vectors may be used to transform monocotyledons, preferably grass species such as ryegrasses (*Lolium* species) and fescues (*Festuca* species), even more preferably perennial ryegrass (*Lolium perenne*), Italian ryegrass (*Lolium multiflorum*) and hybrid ryegrass (*Lolium* x *boucheanum*), including forage- and turf-type cultivars.

Techniques for incorporating the constructs, vectors and chimeric genes of the present invention into plant cells (for example by transduction, transfection or transformation) are known to those skilled in the art. Such techniques include *Agrobacterium*-mediated introduction, electroporation to tissues, cells and protoplasts, protoplast fusion, injection into reproductive organs, injection into immature embryos and high velocity projectile introduction to cells, tissues, calli, immature and mature embryos. The choice of technique will depend largely on the type of plant to be transformed. Other considerations include the ease of transformation, type of tissue and number of gene inserts required.

Cells incorporating the constructs, vectors and chimeric genes of the present invention may be selected, as described above, and then cultured in an appropriate medium to regenerate transformed plants, using techniques well known in the art. The culture conditions, such as temperature, pH and the like, will be able to be determined without undue experimentation by the person skilled in the art. The resulting plants may be reproduced, either sexually or asexually, using methods known in the art, to produce successive generations of transformed plants.

In a further aspect of the present invention there is provided a plant cell, plant, plant seed or other plant part, including, e.g. transformed with, a nucleic acid molecule, construct, vector or chimeric gene of the present invention.

The plant cell, plant, plant seed or other plant part may be from any suitable species, including monocotyledons, dicotyledons and gymnosperms. In a preferred embodiment the plant cell, plant, plant seed or other plant part is from a monocotyledon, preferably a grass species, more preferably a ryegrass (*Lolium* species) or fescue (*Festuca* species), even more preferably perennial ryegrass (*Lolium perenne*), Italian ryegrass (*Lolium multiflorum*) and hybrid ryegrass (*Lolium* x *boucheanum*), including both forage- and turf-type cultivars.

The present invention also provides a plant, plant seed or other plant part derived from a plant cell of the present invention. The present invention also

provides a plant, plant seed or other plant part derived from a plant of the present invention.

In a further aspect of the present invention there is provided a low allergy plant including a nucleic acid molecule, construct, vector or chimeric gene of the present invention. In a preferred embodiment, the low allergy plant is a ryegrass or fescue.

In a further aspect of the present invention there is provided a method of modifying gene expression in pollen, said method including introducing into a plant cell an effective amount of a nucleic acid molecule, construct, vector or chimeric gene according to the present invention.

By "an effective amount" is meant an amount sufficient to result in an identifiable phenotypic trait in the plant cell, or a plant, plant seed or other plant part derived therefrom. Such amounts can be readily determined by an appropriately skilled person, taking into account the type of plant, the route of administration and other relevant factors. Such a person will readily be able to determine a suitable amount and method of administration. See, for example, Sambrook *et al*, 1989, the relevant disclosure of which is incorporated herein by reference.

In a preferred embodiment of this aspect of the present invention, the nucleic acid molecule according to the present invention is used to direct the pollen-specific expression of a further nucleic acid molecule to down-regulate the expression of pollen allergens in the plant. Down-regulation may be achieved *via* any one of a range of known techniques, readily available to a person skilled in the art, including for example antisense and sense suppression technology, and other gene silencing technologies such as *via* the use of interfering RNA. Preferably, the allergen is selected from the major pollen allergens *Lol p* 1 and *Lol p* 2. The sequence of the further nucleic acid molecule may be in either a sense or antisense orientation when operably linked with the nucleic acid molecule of the present invention. Alternatively, the further nucleic acid molecule may be incorporated as multiple copies, in either orientation, in a range of possible RNAi-

generating constructs. RNAi technology has been described in a wide range of publications including, for example, Fire, A. *et al* (1998); Caplan, N. *et al* (2000); US Patent 6,506,559; International patent applications WO 99/53050 and WO 99/49029; and US patent 6,573,099.

Using the methods and materials of the present invention, genes may be targeted for expression in pollen, or the expression of pollen-specific genes may be modified. For example, gene expression may be facilitated in pollen by placing a copy or copies of a further nucleic acid molecule, for example the gene to be expressed or a fragment thereof, operably under the control of the nucleic acid molecule according to the present invention. Furthermore, a nucleic acid molecule of the present invention may be used to introduce a further nucleic acid molecule, for example a gene or fragment thereof, into a plant for specific purposes such as introducing male sterility. Alternatively, decreased expression of an endogenous pollen-specific gene may be achieved by placing a sense or antisense nucleic acid molecule or dsRNA or small interfering RNA (siRNA) derived from the gene operably under the control of the nucleic acid molecule according to the present invention.

In a further aspect of the present invention there is provided a method of producing a plant with reduced male fertility compared with a wild type plant, said method including introducing into the plant a nucleic acid molecule of the present invention in combination with a further nucleic acid molecule capable of modulating male fertility. Preferably the plant is a male sterile plant. In a preferred embodiment, the further nucleic acid molecule may be capable of modifying pollen development, even more preferably the further nucleic acid molecule may be involved in and is preferably a gene or a fragment thereof critical to pollen development. In a further preferred embodiment, the expression of the further nucleic acid molecule may result in cell death at the site of expression. In a further preferred embodiment the further nucleic acid molecule may encode the bacterial ribonuclease barnase or a fragment thereof. The use of the nucleic acid molecule according to the present invention may enable the specific expression of the relevant gene in pollen, reducing any unwanted side-effects of expression in other plant tissues.

In a further aspect of the present invention there is provided a plant with reduced male fertility compared with a wild type plant, preferably a male sterile plant, produced according to the methods according to the present invention. Such plants may be used to develop a transgene containment system by reducing pollen fertility. Furthermore, such plants may be used in hybrid seed production.

In a further aspect of the present invention there is provided a preparation for transforming a plant including a nucleic acid molecule according to the present invention. The preparation may contain vectors or other constructs to facilitate administration to and/or transformation of the plant with the nucleic acid molecule.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the Figures:

Figure 1 shows sequence of genomic clone (SEQ ID NO:1) showing putative promoter (SEQ ID NO:2) and coding regions. Italics (and partially bold): 952 bp of genomic sequence (SEQ ID NO:3); doubly underlined: coding sequence; bold italics, dashed underlined: primer D21pr1L; bold italics: primer D21pr1R (partially doubly underlined). Also shown is the *Sac*I restriction site (bold) and several stop codons in the coding region of the gene (bold underlined).

Figure 2 shows the analysis of promoter elements of the 952 bp genomic region of the pollen-specific promoter from perennial ryegrass; *Zm13* Q element (solid arrowheads), LAT52 element (outlined arrowheads) and GTGANTG10 element (hatched arrowhead).

Figure 3 shows chimeric vectors containing the 952 bp genomic region. **A** pLp952:GUS fusion vector driven by the 952 bp genomic region pBS-260gn (Hamilton *et al.* 1992) was used as the basis for the construction of the promoter-reporter cassette for plant cell transformation using PEG-mediated transformation

techniques. PBS-260gn contains the GUS reporter gene (Jefferson *et al.* 1987) and the nopaline synthase (nos) terminator sequence. **B** Vector containing *Lol p* 1 in an antisense orientation driven by the 952 bp genomic region (pLP2-asLolp1). **C** Vector containing *Lol p* 2 in an antisense orientation driven by the 952 bp genomic region (pLP2-asLolp2).

Figure 4 shows chimeric vectors for gene silencing based on the formation of double-stranded RNA using the 952 bp genomic region of the pollen-specific promoter from perennial ryegrass from the present invention. **A** Vector containing inverted repeats of ca. 200 bp fragment of *Lol p* 1 with LpCCR1 intron. **B** Vector containing inverted repeats of ca. 200 bp fragment of *Lol p* 2 with LpCCR1 intron. **C** Vector containing inverted repeats of combined ca. 200 bp fragments of *Lol p* 1 and *Lol p* 2 with LpCCR1 intron.

Figure 5 shows steps involved in the generation of transgenic tobacco using direct PEG-mediated gene transfer. **A** tobacco protoplasts. **B** Callus regenerated from tobacco protoplasts. **C** Putative transgenic tobacco plantlets on selective medium. **D** Putative transgenic tobacco on root-inducing medium.

Figure 6A shows PCR analysis of transgenic tobacco plants containing the Lp952GUS construct using GUS specific (*gusA*) primers **B**. Southern hybridisation of the PCR positive plants showing the stable integration of *gusA* (Probe:*gusA*).

Figure 7 shows GUS histochemical staining of pollen collected from transgenic tobacco plants containing the Lp952GUS construct.

Figure 8 shows steps involved in the generation of transgenic ryegrass to down-regulate the expression of pollen allergens using used to direct the pollen-specific expression of a gene to down-regulate the expression of pollen allergens. **A** Immature perennial ryegrass inflorescence. **B – F** Development of callus from immature perennial ryegrass inflorescence. **G** Immature inflorescence-derived callus spread on filter disc ready for particle bombardment. **H** particle delivery system. **I, J** Regenerating perennial ryegrass plantlets on selective medium. **K** Putative transgenic perennial ryegrass plantlets on root-inducing medium. **L**

Putative transgenic perennial ryegrass plants under containment glasshouse conditions.

EXAMPLE 1

Cloning of a Novel Promoter

In one embodiment of the present invention, a promoter sequence was isolated from a ryegrass gene. A 3.9 kb fragment of genomic sequence was isolated from a Lambda-DASH II (Stratagene) genomic library constructed from four-week-old perennial ryegrass (*Lolium perenne* L.) cv. Barlano after hybridisation screening of the genomic library with a *Lol p* 2 cDNA sequence. Positive plaques from the tertiary screen were amplified and purified phage DNA was isolated. The genomic region was fully sequenced (Figure 1) and found to contain 3.3 kb including approximately 2.7 kb 5' promoter region and 567 bp of gene sequence which has an ORF of 366 bp and encodes a small protein of 122 amino acids. Promoter elements of the 952 bp genomic region of the pollen-specific promoter from perennial ryegrass are shown (Figure 2).

EXAMPLE 2

Construction of Chimeric Gene Vectors

A PCR product containing 952 bp of promoter region (SEQ ID NO:3) was produced using standard PCR conditions. The sequences of the primers follow.

D21pr1L: 5'-AAAAGTGTGCTGGGATGGTG-3' (SEQ ID NO:7)

D21pr1R: 5'-CCATCCAACAAATCCAGAATGGCTTCC-3' (SEQ ID NO:8)

The 952 bp PCR product was purified, subcloned into pGEMTeasy (Promega), and sequenced to check for PCR amplification errors. A construct was made using the above PCR product as a promoter in fusion with the reporter gene β-glucuronidase (GUS) coding sequence (*gusA*) depicted in Figure 3 A.

The 952 bp PCR product was also used to construct vectors containing the pollen allergen encoding sequences *Lol p* 1 (Figure 3 B) and *Lol p* 2 (Figure 3 C) in antisense orientation. These vectors were designed to be capable of silencing the corresponding endogenous genes.

Additionally, gene silencing vectors based on the formation of double-stranded RNA are designed and constructed using the 952 bp PCR product as the regulatory element and short inverted repeats of *Lol p* 1, *Lol p* 2 and *Lol p* 1+*Lol p* 2 separated by a perennial ryegrass intron (Figure 4).

EXAMPLE 3

The Generation of Transgenic Tobacco Plants for Analysis of Expression Patterns Directed by Novel Promoter

The chimeric GUS fusion vector of Example 1 was transgenically expressed in the heterologous system, tobacco, in order to assess the expression pattern directed by the 952 bp genomic region. Transgenic tobacco plants were generated by PEG mediated direct gene transfer (DGT) of tobacco protoplasts as described in detail below.

A. Isolation of mesophyll protoplasts from tobacco shoot cultures

Fully expanded leaves (2-4) of a 6 week-old shoot culture were placed under sterile conditions in a 9 cm plastic culture dish containing 12 ml enzyme solution [1.0% (w/v) cellulase "Onozuka" R10 and 1.0% (w/v) Macerozyme® R10]. The leaves were wetted thoroughly with enzyme solution and the mid-ribs removed. The leaf halves were cut into small pieces and incubated overnight (14-18 h) at 25°C in the dark without shaking.

The protoplasts were released by gently pipetting up and down, and the suspension poured through a 100 µm stainless steel mesh sieve on a 100 ml glass beaker. The protoplast suspension was mixed gently, distributed into two 14 ml sterile plastic centrifuge tubes and carefully overlayed with 1 ml W5 solution (Spangenberg *et al* 1988). After centrifugation for 5 min. at 70 g (Clements Orbital

500 bench centrifuge, swing-out rotor, 400 rpm), the protoplasts were collected from the interphase and transferred to one new 14 ml centrifuge tube. 10 ml W5 solution were added, the protoplasts resuspended by gentle tilting the capped tube and pelleting, as before. The protoplasts were resuspended in 5-10 ml W5 solution and the yield determined by counting a 1:10 dilution in a haemocytometer.

B. Direct gene transfer to protoplasts using polyethylene glycol

The protoplasts were pelleted [70 g at 400 rpm for 5 min.] and resuspended in transformation buffer to a density of 1.6 x 10^6 protoplasts/ml. Care was taken to carry over as little as possible W5 solution into the transformation mix. Samples (300 µl) of the protoplast suspension (ca. 5 x 10^5 protoplasts) were aliquoted in 14 ml sterile plastic centrifuge tubes, and 30 µl of transforming DNA were added. After carefully mixing, 300 µl of PEG solution (Spangenberg et al 1988) were added and mixed again by careful shaking. The transformation mix was incubated for 15 min. at room temperature with occasional shaking. 10 ml W5 solution were gradually added, the protoplasts pelleted [70g at 400 rpm for 5 min.] and the supernatant removed. The protoplasts were resuspended in 0.5 ml K3 medium (Spangenberg *et al* 1988), ready for cultivation.

C. Culture of protoplasts, selection of transformed lines and regeneration of transgenic tobacco plants

Approximately 5 x 10^5 protoplasts were placed in a 6 cm petri dish. Pre-warmed (melted and kept in a water bath at 40-45°C) 1:1 mix of K3:H medium (4.5 ml) (Spangenberg *et al* 1988) containing 0.6% SeaPlaque™ agarose were added and, after gentle mixing, allowed to set.

After 20-30 min the dishes were sealed with Parafilm® and the protoplasts were cultured for 24 h in darkness at 24°C, followed by 6-8 days in continuous dim light (5 µmol m^{-2} s^{-1}, Osram L36 W/21 Lumilux white tubes), during which time first and multiple cell divisions occurred. The agarose containing the dividing protoplasts was cut into quadrants and placed in 20 ml of A medium (Spangenberg *et al* 1988) in a 250 ml plastic culture vessel. The corresponding selection agent was added to a final concentration of 50 mg/l kanamycin sulphate

(for *npt2* expression) or 25 mg/l hygromycin B (for *hph* expression). Alternatively, selection may be carried out using 20 mg/l phosphinotricin (for *bar* expression). Samples were incubated on a rotary shaker with 80 rpm and 1.25 cm throw at 24°C in continuous dim light.

Resistant colonies were first seen 3-4 weeks after protoplast plating, and after a total time of 6-8 weeks protoplast-derived resistant colonies (when 2-3 mm in diameter) were transferred onto MS morpho medium (Spangenberg *et al* 1988) solidified with 0.6% (w/v) agarose in 12-well plates and kept for the following 1-2 weeks at 24°C in continuous dim light (5 µmol m^{-2} s^{-1}, Osram L36 W/21 Lumilux white tubes). During this time, calli proliferated and reached a size of 8-10 mm; shoots that were rooted on MS hormone free medium (Spangenberg *et al* 1988) differentiated and transgenic tobacco plants were recovered. (Figure 5).

The putative transgenic tobacco plants were screened by PCR and Southern hybridisation analysis. The PCR screening was undertaken using *gusA* specific primers for the initial identification of transformed plants (Figure 6A). The presence of the *gusA* gene was demonstrated by PCR-amplification of a 270 bp fragment using the forward primer:

5'-CTTTAACTATGCCGGGATCCATCG-3' (SEQ ID NO:9)

and the reverse primer:

5'-TAACCTTCACCCGGTTGCCAGAGG-3' (SEQ ID NO:10).

The PCR positive transgenic plants were then analysed by Southern hybridisation to show stable integration of the transgene (Figure 6B). For Southern hybridisation analysis, genomic DNA was extracted from lyophilised plant material using a CTAB-based protocol. DNA samples (10 – 15 µg) were digested with *Bam*HI, *Hin*dIII, *Eco*RI, or *Xho*I restriction enzymes. The resulting DNA fragments were separated on a 1% agarose gel and transferred to Hybond N (Amersham Pharmacia Biotech) membranes. Hybridisation was performed according to the manufacturer's instructions and incorporation of DIG-dUTP into DNA probes and

detection of bound probes was performed using the DIG Luminescent Detection Kit (Roche Diagnostics Cat. No 1363514) following the supplier's protocol. Hybridisation conditions were: 4 x SSC, 50% (v/v) formamide, 0.1% (w/v) N-lauroyl-sarcosine, 0.02% (w/v) SDS, and 2% (v/v) blocking solution at 42°C. Membranes were washed twice in 2 x SSC/0.1% (w/v) SDS for five minutes at 25°C then 0.2 x SSC/0.1% (w/v) SDS followed by 0.1 x SSC/0.1% (w/v) SDS, both for fifteen minutes at 68°C.Southern positive plants were transferred to soil and grown under glasshouse conditions until flowering.

EXAMPLE 4

Assay Promoter Activity in Plant Cells Under Stable Conditions

Tissue samples were collected from the Southern positive plants and screened by histochemical GUS assays to assess the expression pattern of the 952 bp *Lolium perenne* promoter. Expression of the *gusA* reporter gene was observed exclusively in the pollen grains of the transgenic tobacco plants containing the 952 bpGUS fusion (Figure 7).

These results indicate that the 952 bp region of *Lolium perenne* genomic sequence confers strong pollen-specific expression to the *gusA* gene coding sequence and is thus a pollen-specific promoter that represents an excellent candidate for applications requiring targeted gene expression to pollen cells such as, for example, transgene containment and/or the down-regulation of pollen allergen genes and/or induction of male sterility.

EXAMPLE 5

The Generation of Transgenic Perennial Ryegrass Plants for Down-Regulation of Pollen Allergens Using Chimeric Genes Under Control of the Novel Pollen-Specific Promoter

The steps in the production of transgenic ryegrass plants for the down-regulation of main pollen allergens *Lol p* 1 and *Lol p* 2, using chimeric genes under control of pollen-specific promoter, are shown in Figure 8.

A. Production of target material for biolistic transformation

Multi-tillered, well-established donor plants, generated from seedlings, are vernalised at less than 10 °C (minimum 4 °C) under an 8 hour day length for a period greater than 8 weeks. Flowering of plants is induced by growing at 24 °C under an increased day length of greater than 12 hours (optimum 16 hours).

Plants are monitored daily after the first 2 – 3 days of induction and floral tillers identified for harvest. Target immature inflorescences should be whitish in colour and no longer than 0.5 mm in length.

Selected floral tillers are harvested by cutting just below the base node and before the youngest leaves. The tillers are stripped of all leaf material and collected in one clean plastic vessel. For sterilisation, the collection vessel is filled with a sterilisation solution containing 5% available chlorine with 5 drops of Tween 20 for every 100 ml to ensure adequate sterilisation of plant material. Sterilisation of tillers is achieved by medium-to-high speed shaking for 20 minutes on a bench top platform shaker.

Subsequently, tillers are rinsed thoroughly with sterile distilled water and transferred in batches of 8 – 10 on to sterilized sheets of A5 paper.

Immature inflorescence are prepared, cut free from the base node and transferred to calli induction medium, LP5 [MS Macro, MS Micro, MS Vitamins (MS hormone free) with 5 mg/l 2,4-Dichlorophenoxyacetic acid and 30 g/l maltose] + 250 mg/l cefotaxime. If the inflorescence in not intact (most likely in 2 pieces due to the cut), pieces may be plated individually.

All dishes are sealed with parafilm and incubated in the dark at 24°C for up to 8 weeks to induce calli development.

B. Preparation of embryogenic calli for microprojectile bombardment

Embryogenic, friable, yellowish calli (1-2), derived from plated immature inflorescences, are transferred onto media-covered filter paper. Calli are squashed to yield an even, fine layer of cells across the disc surface and incubated for 4-6 hours at room temperature to prepare cells for bombardment.

C. Preparation of particles for microprojectile bombardment

To a sterile 1.5 ml Eppendorf tube, 10 µl transforming DNA (1 µg/µl; gene of interest), 10 µl selectable marker (1 µg/µl), 100 µl gold particle solution (60 mg/ml), 100 µl 2.5 M $CaCl_2$ and 40 µl 100 mM spermidine are added, vortexed for 1 minute and allowed to sediment for 1 minute. The supernatant is removed, particles are resuspended in 900 µl 100% (w/v) filter-sterilized ethanol and mixed. The washed particles are sedimented and the washing step is repeated. The particles are then resuspended in 200 µl 100% (w/v) filter-sterilized ethanol.

D. Microprojectile bombardment of immature inflorescence-derived calli using a Biorad Particle Delivery System

Microcarriers are soaked in 100% (w/v) isopropanol for 30 minutes, air-dried, embedded in autoclaved macrocarriers and transferred to a U.V.-sterilized tip box containing dehydrated silica gel. Prepared particles (20 µl) are then loaded to the centre of a microcarrier and air-dried. Particles prepared as detailed in Section C above, are then delivered to the target material, following the particle delivery systems manufacturer's instructions.

Plates are subsequently sealed with parafilm and incubated in the dark at 25°C overnight.

E. *Agrobacterium*-mediated transformation of embryogenic callus

The embryogenic material obtained as described above can alternatively be used for *Agrobacterium*-mediated transformation. Sample tissues are inoculated

by vacuum infiltration with an *Agrobacterium* suspension ($O.D._{600}$ 0.3-1.0) in a modified liquid AA medium (Spangenberg *et al* 1995) (AA major inorganic salts, AA amino acids and vitamins, 2% (w/v) sucrose, 3% (w/v) sorbitol, 0.2mg/L kinetin, 0.1mg/L gibberellic acid, 6.8 µM 2,4-D and 100µM acetosyringone). Sample tissues are then transferred to sterile dry filter paper for 3 days in the dark at 21°C. Selection and regeneration are as described in Section F, below.

F. Selection of transformed calli and regeneration of transgenic ryegrass plants

Filter paper discs containing bombarded calli are transferred to solidified proliferation media, LP3 [MS Macro, MS Micro, MS Vitamins (MS hormone free) with 3 mg/l 2,4-Dichlorophenoxyacetic acid and 30 g/l maltose] to induce growth of cells. Plates are sealed with parafilm and incubated in the dark at 25°C for 48 hours. The filter paper discs are then transferred to solidified selection media, LP3 + 100 mg/l hygromycin + 250 mg/l cefotaxime and incubated in the dark at 25°C. After 2 weeks, the filter paper discs are transferred to solidified regeneration medium, MSK (Spangenberg *et al* 1995) + 100 mg/l hygromycin + 250 mg/l cefotaxime and incubated under direct light at 25°C under fluorescent light conditions (16 hr light/8 hr dark photoperiod; 55 µmol m^{-2} sec^{-1}) to encourage shoot and root development.

Hygromycin-resistant (Hyg^r) shoots with developed roots are then transferred to shoot elongation medium, MSO [MS Macro, MS Micro, MS Vitamins (MS hormone free) with 30 g/l maltose] + 250 mg/l cefotaxime, and incubated at 25° C under an 8h photoperiod until root systems become established. Finally, the plants are transferred to soil and maintained under containment glasshouse conditions.

G. Molecular analysis of transgenic ryegrass plants for down-regulation of pollen allergens using chimeric genes under control of pollen specific promoter

Initial screening of the putative transgenic perennial ryegrass plants is achieved by PCR analysis.

The PCR-positive transgenic plants are then analysed by Southern hybridisation to show stable integration of the transgene. Genomic DNA is extracted from lyophilised plant material using a CTAB-based protocol. DNA samples (10 – 15 µg) are digested with *Bam*HI, *Hind*III, *Eco*RI, or *Xho*I restriction enzymes. The resulting DNA fragments are separated on a 1% agarose gel and transferred to Hybond N (Amersham Pharmacia Biotech) membranes. Hybridisation is performed according to the manufacturer's instructions and incorporation of DIG-dUTP into DNA probes and detection of bound probes is performed using the DIG Luminescent Detection Kit (Roche Diagnostics Cat. No 1363514) following the supplier's protocol. Hybridisation conditions are: 4 x SSC, 50% (v/v) formamide, 0.1% (w/v) N-lauroyl-sarcosine, 0.02% (w/v) SDS, and 2% (v/v) blocking solution at 42°C. Membranes are washed twice in 2 x SSC/0.1% (w/v) SDS for five minutes at 25°C then 0.2 x SSC/0.1% (w/v) SDS followed by 0.1 x SSC/0.1% (w/v) SDS, both for fifteen minutes at 68°C. Southern-positive plants are transferred to soil and grown under glasshouse conditions until flowering.

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis, total proteins are extracted by grinding mature anthers from ryegrass plants in PBS buffer (10 mM phosphate buffer, pH 7.2; 150 mM NaCl) containing 1 mM PMSF followed by centrifugation at 14,000 rpm for 20 minutes at 4°C. Soluble proteins in the supernatant are quantified by Bio-Rad assay (Bio-Rad). Proteins are separated on SDS/15% PAGE gels on a Mini-Protean II system (Bio-Rad). The gels are either stained with Coomassie brilliant blue R250 or used in western blot analysis.

For western blotting, the proteins separated by SDS-PAGE are transferred onto nitrocellulose membrane. The blots are probed with primary antibodies or

human sera overnight at 4°C. The primary antibodies, including polyclonal rabbit anti-*Lol p* 1 or polyclonal rabbit anti-*Lol p* 2, are diluted 1:1000 by using PBS. The sera of patients allergic to grass pollen are used at a dilution of 1:10. Binding of polyclonal antibodies is detected with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) at a dilution of 1:1000, while binding of human sera is detected with mouse anti-human IgE antibodies conjugated to alkaline phosphatase (Southern Biotech) at a dilution of 1:1000. Secondary antibody detection is carried out for 2 hours at 25°C. The colour reaction is developed by using an alkaline phosphatase conjugate substrate kit (Bio-Rad). Equal loading of total proteins from samples of transgenic and non-transformed control plants for the western analysis is ensured by quantifying total protein content and Bio-Rad assay staining of replicate gels for each extraction.

EXAMPLE 6

The Generation of Transgenic Plants for Induction of Male Sterility and/or Transgene Containment Using Chimeric Genes Under Control of the Novel Pollen-Specific Promoter

Transgenic male sterile plants are produced by introducing into the plant the nucleic acid molecule of the present invention in combination with a gene capable of modulating male fertility. Established methods for gene transfer to plants are used for the production of transgenic plants as described in 'Gene Transfer to Plants' I. Potrykus and G Spangenberg, Springer Lab Manual, 1995, ISBN 3-540 58406-4, and/or as set forth in Examples 3 and 5, above.

The gene used to modulate male fertility is a gene critical to pollen development and/or germination. Suitable genes include, for example, genes encoding pollen callose synthase, pollen tubulin, pollen actin or some other pollen-expressed 'house-keeping genes'. The chimeric genes transferred into the plant for induction of male sterility lead to decreased expression of the endogenous plant pollen-expressed gene, whether encoding pollen callose synthase, pollen tubulin, pollen actin or some other pollen-expressed 'house-keeping gene'. This is achieved by placing an antisense nucleic acid molecule or dsRNA or small

interfering RNA (siRNA), derived from the plant pollen-expressed gene, operably under the control of the promoter according to the present invention.

A gene capable of modulating male fertility for the production of transgenic male sterile plants may also be a gene the expression of which results in cell death at the site of expression. Such genes include the gene encoding the bacterial secreted ribonuclease, barnase, derived from *Bacillus amyloliquefaciens*. The expression of a transferred chimeric gene that includes the coding sequence for the ribonuclease barnase from *Bacillus amyloliquefaciens,* operably under control of the nucleic acid molecule according to the present invention, leads to the specific-expression of the barnase gene in pollen, thereby reducing any unwanted side-effects of expression of barnase in other plant tissues.

EXAMPLE 7

The Restoration of Male Fertility in Male Sterile Transgenic Plants Using Chimeric Genes Under Control of the Novel Pollen-Specific Promoter for Hybrid Production

Transgenic male fertile plants are produced by introducing into the plant the nucleic acid molecule of the present invention in combination with a gene capable of reverting the action of a gene that leads to male sterility. Established methods for gene transfer to plants are used for the production of transgenic plants as described in 'Gene Transfer to Plants' I. Potrykus and G Spangenberg, Springer Lab Manual, 1995, ISBN 3-540 58406-4, and/or as set forth in Examples 3 and 5, above.

The gene capable of restoring male fertility of transgenic male sterile plants – generated by the pollen-specific expression of a gene which results in cell death at the site of expression, including the gene encoding the bacterial secreted ribonuclease barnase from *Bacillus amyloliquefaciens* – is a gene that counteracts the effect of the male sterility gene. The barstar gene from *Bacillus amyloliquefaciens,* which encodes the inhibitor of the bacterial secreted ribonuclease barnase from *Bacillus amyloliquefaciens*, is used as male fertility restorer gene. Chimeric genes that include the barstar gene from *Bacillus amyloliquefaciens*, operably under control of the nucleic acid molecule according

to the present invention, lead to the specific expression of the barstar gene in pollen. Crosses of transgenic male sterile plants expressing the barnase gene operably under control of a pollen-specific promoter, including the nucleic acid molecule according to the present invention, are made with pollen from transgenic male fertile plants expressing the barstar gene operably under control of the nucleic acid molecule according to the present invention. Offspring recovered from these crosses lead to fertile hybrid plants.

Those skilled in the art will appreciate that the invention described above is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and products referred to or indicated in this specification, individually or collectively, and any and all combinations of two or more of said steps or features.

Reference to any prior art in the specification is not, and should not be taken as, an acknowledgement or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other jurisdiction.

REFERENCES

Caplan, N. *et al* (2000); Gene 252:95-105

Fire, A. *et al* (1998); Nature 391:806-811

Hamilton DA, Roy M, Rueda J, Sindhu RK, Sanford J, Mascarenhas JP (1992) Dissection of a pollen-specific promoter from maize by transient transformation assays. *Plant Mol. Biol.* 18:211 – 218.

Jauhar PP (1993) Cytogenetics of the *Festuca-Lolium* complex. Relevance to breeding. In: Frankel R, Grossman M, Linskens HF, Maliga P, Riley R (eds) Monographs on theoretical and applied genetics, vol 18. Springer, Berlin Heidelberg New York, 243 pp.

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, *EMBO J.* 6:3901-3907.

Potrykus I, Spangenberg G (eds.) (1995) Gene transfer to plants, Laboratory Manual, Springer Verlag, Heidelberg

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning - a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, New York, 1989.

Siegel MR, Latch GCM, Johnson MC (1985) *Acremonium* fungal endophytes of tall fescue and perennial ryegrass: significance and control. *Plant. Dis.*, 69: 179-183.

Spangenberg G, Wang ZY, Potrykus I (1998) Isolation, culture and plant regeneration from protoplasts, In Cell Biology: A Laboratory Handbook, Second Edition, Vol 1, Academic Press

Spangenberg G, Wang ZY, Wu X, Nagel J, Potrukus I (1995), Transgenic perennial ryegrass (Lolium perenne) plants from microprojectile bombardment of embryogenic suspension cells, Plant Science 108:209-217